Low Temp. Sci. Ser. B 36: 31-38, 1978

Development of a whole plant from an excised strawberry runner apex frozen to −196°C[1]

Akira SAKAI, Megumi YAMAKAWA[2], Daijiro SAKATA[2],
Takashi HARADA[2] and Toshiro YAKUWA[2]
酒井　昭・山川　恵・坂田大二郎
原田　隆・八鍬利郎

Abstract Excised runner apices from strawberry which were floated on 0.5 ml of freezing solution including 3% sucrose and 10 to 16% DMSO in a spitz tube were immersed in liquid nitrogen (cooling rate: 420°C/min) after prefreezing to −40°C and were then rewarmed rapidly. Only about 15% of these treated apices initiated shoot development in the light on MS cultural medium. To increase the survival rate of the apices frozen to the temperature of liquid nitrogen, excised runner apices on a coverglass were rapidly frozen from room temperature in liquid nitrogen (cooling rate: 10^5°C/min) after prefreezing to −20 to −30°C in the presence of 16% DMSO and were then rewarmed very rapidly by direct immersion in MS cultural solution at 40°C. About 60 to 80% of the runner apices frozen to −196°C developed normal shoots in the light on the MS cultural medium.

Introduction

Cryopreservation of genetic resources is increasing in importance as an international co-operative biological programme. The development of a routine freeze-preservation method for plant culture lines is also required. Recently, freeze-preservation in liquid nitrogen has been successfully applied to plant cultures of some higher plant species (1, 5, 6, 7, 19). However, regeneration of plants from cultured cells that had been cooled down to the temperature of liquid nitrogen has been observed with only a limited number of species (2, 5, 7).

On the other hand, shoot apex cultures are routinely used to propagate many species and give rise to plants that are pathogen free (4). Small explants from shoot tips, as recommended for the recovery of virus-free plants, consist of the apical meristematic dome, together with one to a few leaf primordia. The successful development of a whole plant from an excised shoot apex after freezing to −196°C was first demonstrated with a carnation plant by Seibert and Wetherbee (17, 18). However, using their cooling method, we obtained little or no survival using the apices from strawberry runners

[1] Received for publication October 31, 1978. Contribution No. 1987 from the Inst. Low Temp. Sci.
[2] Dept. of Horticult. Sci., Hokkaido University

and asparagus seedlings.

This study was designed to find some factors contributing to the survival of apices of strawberry runners frozen to the temperature of liquid nitrogen.

Material and Methods

Runners, 15 cm long, were cut from strawberry plants (*Fragaria* × *ananassa* Duch, cv. 'Hokowase') during summer and autumn. These samples, enclosed in a polyethylene bag, were placed at 4°C for 3 days. During this cold treatment, an 8-hr photoperiod (1,500 lux) was provided by white fluorescent and incandescent lamps having a combined intensity of 1,500 lux at sample level. However, a noticeable hardening effect was not observed in this treatment. After the treatment, runner apices 0.5 to 1.0 mm long were excised from surface sterilized runner tips. The excised apices consisted of the apical dome surrounded by 1 to 2 of the leaf primordia, and a conical piece of subapical tissue. Sample apices were floated on 0.25 ml of 6% sucrose solution in a graduated spitz tube (15 mm diameter, 110 mm long). An additional 0.25 ml of DMSO solution at twice the final desired concentration was gradually added and sufficiently mixed. After standing at room temperature (25 to 30°C) for 1 hr, sample apices were immersed in an ethanol bath at −8°C. To induce freezing in the sample suspension, the bottom of the tube was cooled with Dry Ice for a few seconds. Thus, frozen suspensions were successively cooled down at 3-min intervals to the test temperatures of −10, −15, −23, −30, −40 and −50°C, respectively. Some suspensions frozen at these temperatures for 3 minutes were then immersed in liquid nitrogen (cooling rate: 420°C/min). Sample suspensions held at each test temperature or immersed in liquid nitrogen for 5 min after prefreezing to these temperatures (9, 10, 13) were rewarmed rapidly in water at 40°C (rewarming rate: 500°C/min). In most of the experiments sample apices were cooled and rewarmed very rapidly. Apices treated with DMSO solution were placed on a coverglass and the surrounding solution was removed. These samples were very rapidly cooled by direct immersion in liquid nitrogen (cooling rate: 10^5°C/min) with or without prefreezing and were then rapidly rewarmed by immersion in the MS cultural solution containing 3% sucrose at 40°C. Thawed apices were washed twice in the cultural solution and then planted on the MS growing medium solidified with 0.6% agar.

The temperature of the material was determined with 0.1 mm copper-constantan thermocouples and was recorded with an oscilloscope. The cooling rate of frozen suspensions immersed in liquid nitrogen was represented as the time required from the temperature to fall from −10 to −100°C.

Apices which sustained freezing injury lost their original light green color. Apices which remained green or which showed signs of growth 10 days after thawing were termed surviving apices. Developing apices were defined as those which initiated shoot development 20 days after thawing.

Results

In the strawberry apices cooled slowly in the presence of 10% DMSO, survival decreased with decreasing temperatures down to −50°C (Fig. 1). Sample apices were immersed in liquid nitrogen after prefreezing from −10 to −50°C in the presence of DMSO, and were rewarmed rapidly in water at 40°C. The apices prefrozen above −20°C were all killed regardless of rewarming methods. In the apices prefrozen to −30 and −40°C, the survival rate increased with decreasing temperatures down to −40°C, reaching 25%. However, in the apices immersed in liquid nitrogen after prefreezing to −40 °C, only about 15% initiated development even in the presence of 16% DMSO (Fig. 2). Though about 90% of carnation shoot apices prefrozen at −40°C developed normal shoots after immersion in liquid nitrogen (unpublished data).

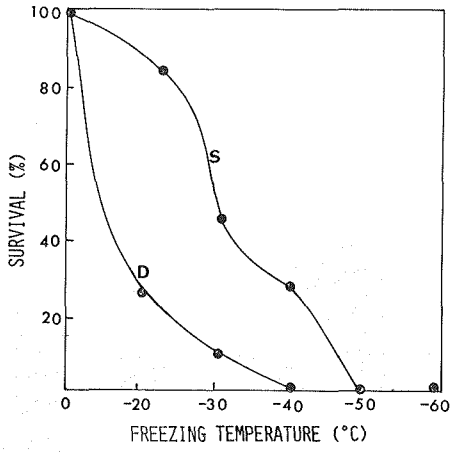

Fig. 1. Survival of strawberry runner apices frozen slowly to various temperatures in the presence of 10% DMSO.

These apices were all rewarmed rapidly in water at 40°C, and were then planted on the MS cultural medium solidified with 0.6% agar. S, D: Apices remaining green 10 days after thawing (S) and apices initiating development (D) 20 days after thawing.

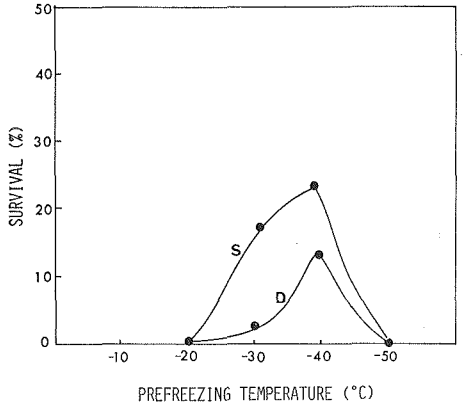

Fig. 2. Survival of runner apices of strawberry immersed in liquid nitrogen following prefreezing to various temperatures in the presence of 10% DMSO.

S, D: Refer to Fig. 1.

In the strawberry runner apex, the apical meristem and leaf primordia were observed to be much more resistant than the subapical tissue. In the strawberry apices, however, a marked difference was not observed between the leaf primordia and apical meristem unlike carnation shoot apices (17, 18).

In carnation apices, Seibert et al. showed 80% survival after freezing to the temperature of liquid nitrogen (17, 18). In their experiments, carnation shoot apices which were floated on 0.5 ml of freezing solution in 50-ml specimen vials were rapidly cooled by pouring liquid nitrogen directly nto the vial, while dipping the vial directly into an open Dewar flask filled with

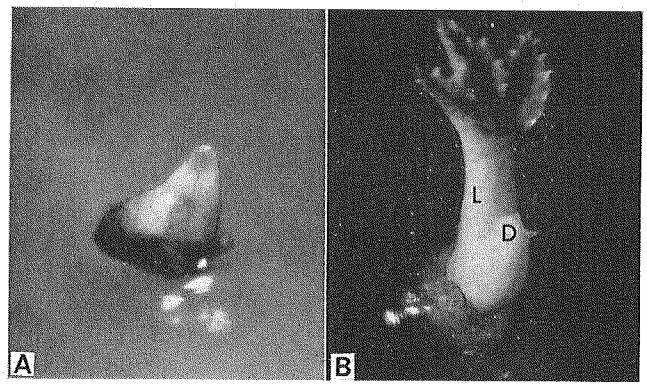

Fig. 3. Apices frozen very rapidly to the temperature of liquid nitrogen.

A: Apex planted on the culture medium immediately after thawing, B: Initiation of development of apex cultured in the light for 20 days after thawing. L: Leaflet, D: Meristem dome.

liquid nitrogen (cooling rate: 400°C/min, a maximum rate: 1,100°C/min). In this cooling method, however, no survival was observed in the strawberry runner apices.

In previous papers (14, 15), we demonstrated that even less hardy cells could survive very rapid cooling to the temperature of liquid nitrogen and rewarming therefrom, provided that they were prefrozen between −10 and −20°C in the presence of cryoprotectants. To obtain much higher survival, sample apices were cooled and rewarmed very rapidly. Apices treated with DMSO solution were placed on a coverglass and the surrounding solution was removed. Then they were cooled very rapidly by direct immersion in liquid nitrogen (cooling rate: 10^5°C/min). These frozen apices were then rewarmed very rapidly by immersion into the MS cultural solution containing 3% sucrose solution at 40°C. Using this method 40 to 60% survival was obtained in the presence of 12 to 16% DMSO solution. And most of the apices frozen in liquid nitrogen had initiated development within 20 days after thawing (Fig. 3). Much higher survival (60 to 80%) was obtained in the apices

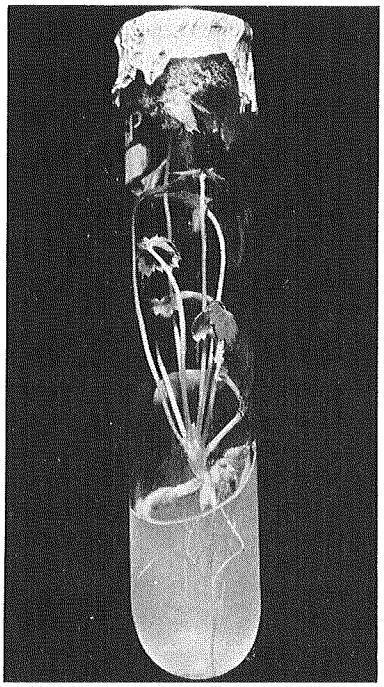

Fig. 4. A plantlet obtained from a strawberry runner apex frozen very rapidly to the temperature of liquid nitrogen.

Treated apex was cultured in the light for 80 days.

cooled very rapidly after prefreezing to −20 to −30°C. A plantlet obtained from a strawberry runner apex frozen very rapidly to the temperature of liquid nitrogen is shown in Fig. 4.

Discussion

Usually 0.5 to 1.0 ml suspension cultures in a spitz tube or ampoules are frozen slowly to the temperatures of liquid nitrogen or immersed in liquid nitrogen after prefreezing to −40°C (19). In the strawberry apices tested a high survival was not obtained by this prefreezing method, though 90% survival was obtained in carnation shoot spices in the presence of 16% DMSO (unpublished data).

The senior author reported that less or non hardy cells could survive immersion in liquid nitrogen by rapid passage through the growth zone of intracellular ice crystals (3), provided that the cells were partially dehydrated by slow freezing in the presence of cryoprotectants (10, 11, 12, 13, 14). Prefreezing reduces greatly the growth rate of intracellular ice crystals formed during rapid cooling (11, 12). The cooling and rewarming rates necessary to survive very rapid cooling to and rewarming from liquid nitrogen vary considerably, depending upon the presence or absence of suspending medium, the amount of medium, the degree of prefreezing and the freezing resistance in the presence of cryoprotectants of the plant materials concerned (11, 12, 13). In general, lower cooling and rewarming rates require much prefreezing. This very rapid cooling method following prefreezing may be applicable to other shoot apices and organ cultures. It is now required to develop a simple and reliable routine method of cryopreservation of plant cell lines, shoot apices and organ cultures.

In many culture cells (6, 15), the optimal concentration of DMSO was observed to be 5 to 8%. Culture cells are treated with DMSO near 0°C to reduce toxicity. However, treatment with 16% DMSO for 1 hr at 25° to 30°C appears to be optimal for the survival and development of runner apices from strawberry. Little or no toxicity was observed in the unfrozen apices treated with 16% DMSO for 1 hr at 30°C. These facts suggest that in the strawberry apices, penetration of DMSO into meristematic cells seems to be considerably more difficult than into cultured cells (15).

In carnation shoot apices, cold treatment at 4°C for 3 days, greatly improves the survival and differentiation rates of excised shoot apices after cooling to −196°C (18). This was not the case with strawberry runners.

The morphological and physiological condition of the plant material prior to freezing greatly influences its ability to survive. We demonstrated that a higher percentage of cells from 5- to 6-day-old sycamore suspension cultures survived freezing to −30°C in the presence of cryoprotectants than did cells at any other stage of growth (19). At this point of development (late lag or early cell division), the cells were known to be small and dense

(20). Bajaj (2) has first reported that globular embryos of haploid pollen-embryo cultures of *Atropa* and *Nicotiana* frozen to the temperature of liquid nitrogen showed higher survival (31%) as compared to early heart-shaped embryos (9%) and late heart-shaped embryos (2%). This low survival of the maturing embryo is attributed to their expanded and differentiated cells in contrast to the highly cytoplasmic, small sized, non-vacuolated and thin walled cells of globular embryos. And Withers (23) has recently demonstrated that the maximal recovery potential of carrot somatic embryo frozen to the temperature of liquid nitrogen was obtained at the late globular and early heart stages (unpublished).

In the strawberry runner apex, the apical meristem and leaf primordia remained viable after freezing, while the subapical tissue stripped off leaf primordia always sustained freezing injury. Meristematic cells in shoot apices of plants are known to be highly cytoplasmic, small sized and less or non-vacuolated cells. Further studies are required to explain more fully differences between the cultural cells of different stages and between the cells of different species as to their susceptibility to injury in the freeze-thaw cycle in the presence of cryoprotectants, and to optimize conditions for each species.

A number of tissue culture techniques have been proposed to preserve selected plant genotypes for extended periods of time. Such cultures are usually maintained by methods involving repeated subculturings (4). However, long-term culturing has undesirable consequences such as contamination, mutation and selection, chromosome abnormalities and loss of morphogenetic potential (21). Therefore, a method to enable their long-term preservation under conditions that would prevent or minimize such changes is desired. Nag and Street (5) first demonstrated that cultured cells from high embryogenic cell lines of carrot, stored in liquid nitrogen for 45 days, initiated embryos and then developed into healthy plants of normal morphology. And no decline in rate of cell survival was observed during storage in liquid nitrogen for over 100 days (5). They also reported that no impairment of morphogenic potential was observed during storage and that examination of meiotic metaphase in large numbers of plants yielded no evidence of any effect of freezing preservation on the karyotype.

Recently we confirmed that apple leaf buds immersed in liquid nitrogen for 23 months still remained alive (16). These facts suggest that it may be possible to establish cell and tissue banks of particular plant genotypes using long-term storage in liquid nitrogen. The value of this technique for the preservation of genetic resources would depend upon the ease with which tissue cultures possessing high propagation potential could be isolated and which are not readily preserved by orthodox methods. Regeneration or morphogenesis of plants derived from culture cells seems to be difficult in many plant species at present. However, shoot apex cultures offer a promising prospect for establishment of plant organ banks to preserve plant genotypes without the associated problems.

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